Elucidating a Biological Role for Chromium at a Molecular Level

JOHN B. VINCENT
Department of Chemistry and Coalition for Biomolecular Products, The University of Alabama, Tuscaloosa, Alabama 35487-0336

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ABSTRACT
Chromium is an essential trace element for mammals and is required for maintenance of proper carbohydrate and lipid metabolism. However, elucidating its function at a molecular level has proved to be problematic. Recent research has revealed that the chromium-binding oligopeptide chromodulin may play a unique role in the autoamplification of insulin signaling. Attempts to develop chromium-containing nutritional supplements and therapeutics are described.

Introduction

Over four decades ago, Walter Mertz and co-workers demonstrated that chromium was an essential trace element for mammals.[1,2] Rats that were fed a specific diet which proved to be chromium deficient developed an inability to respond efficiently to increases in blood glucose. Two materials, acid-hydrolyzed porcine kidney powder and Brewer's yeast, were identified that could reverse the condition when added to the diet. Both were subsequently found to be rich in chromium, and the addition of simple chromic salts (such as $CrCl_3$ or chrome alum) to the diet also reversed the glucose intolerance. The dietary factor necessary to prevent this glucose intolerance was named glucose tolerance factor (GTF). These results have been questioned on occasion over the past 40 years, but very recently the essential aspects of the original studies from the late 1950s and early 1960s have been reproduced.[3,4] The recent studies clearly indicate that very strict environmental control, such as removal of contact with stainless steel, is required to induce chromium deficiency in rats and that the symptoms of deficiency can be prevented by the addition of chromium to the deficient diet. This chromium deficiency can be accentuated by application of stress, such as a high fat content in the diet.[4] For humans, chromium deficiency has unambiguously been observed for patients on total parenteral nutrition, where it results in symptoms similar to those of adult-onset diabetes.[5] Despite these observations, a recent review of the role of metal ions in biological systems by well-respected bioinorganic chemists stated that the set of biological metals "includes magnesium, calcium, all members of the first transition series (excluding scandium, titanium, and chromium) and molybdenum, tungsten, cadmium, and mercury".[6] Why the discrepancy? The matter is simple; if chromium is an essential nutrient, presumably it must interact with (or be bound to) a specific biomolecule(s) and possess a specific function. In other words, there should be a naturally occurring, biologically active form of chromium.[7,8] At the time of the review, almost 40 years after the first report of GTF, no conclusive evidence for an isolable, biologically active form of chromium existed. Three materials have been proposed to be the biologically active form of chromium: "glucose tolerance factor", chromium picolinate, and low-molecular-weight chromium-binding substance (LMWCr). Workers in this laboratory have determined to identify the nature, function, and mode of action of the biologically active form(s) of chromium and has uncovered the basic mechanism by which chromium influences carbohydrate and lipid metabolism and may be related to adult-onset diabetes and cardiovascular disease.

Glucose Tolerance Factor

After the identification of the requirement for chromium, efforts were turned to identifying an active Cr-containing metal−organic species and how it might affect carbohydrate and lipid metabolism. Studies in the early 1960s using epididymal fat and later isolated adipocytes from chromium-deficient rats suggested that the effects of chromium required insulin.[9−11] Unfortunately, from this point several wrong turns were taken. Attempts were made to isolate a biologically active form of chromium from Brewer's yeast and porcine kidney powder. As the material from Brewer's yeast and acid-hydrolyzed porcine kidney powder appeared to be very similar and the yeast material was more easily obtained in appreciable quantities, studies have focused almost exclusively on yeast. The material subsequently isolated from yeast was also termed "glucose tolerance factor", and currently this term is generally understood to refer to the yeast-derived material. [This has resulted in considerable confusion in the literature, especially as several different materials have been termed "GTF"]. In the apparent belief that the active form of chromium would be an acid-stable, heat-stable vitamin, the isolation procedure for the yeast material involved an 18 h hydrolysis in refluxing 5 M HCl (which would have largely destroyed any proteinaceous components or nucleic acids) before the chromium-containing material was purified by chromatography.[12] The isolated product was proposed to be composed of chromic ion, nicotinic acid, and the amino acids glycine, glutamic acid, and cysteine; however, these results have not been reproduced in several laboratories (reviewed in ref 13). Yet, the yeast "GTF" was found to stimulate glucose degradation by epididymal tissue from chromium-deficient rats in the presence of insulin. The ability of "GTF" to stimulate

John B. Vincent is currently Associate Professor and Director of Undergraduate Studies in the Department of Chemistry at The University of Alabama. His research interests include the study of chromium biochemistry, the invention of biomimetic chromium-containing therapeutics, and the development of metalloprotein affinity metal chromatography. He received a B.S. degree from Murray State University in 1984, received a Ph.D. degree from Indiana University in 1988 under the supervision of George Christou, and was a NIH postdoctoral fellow with Bruce Averill at the University of Virginia from 1988 until 1991.

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glucose metabolism can be elucidated from kinetic studies following the rate of glucose metabolism by isolated adipocytes from Cr-deficient rats over a range of insulin and "GTF" concentrations.[14] Analysis of these results in this laboratory showed that "GTF" did not have an intrinsic activity in the cells but was simply acting as a source of chromium, restoring the chromium pool of the cells from the Cr-deficient rats.[15] Once the chromium levels were restored, "GTF" actually inhibits stimulation of glucose metabolism by insulin, presumably by binding insulin. Earlier proposals that GTF's natural function is to bind insulin, enhancing its interaction with insulin receptor, are in error; they were primarily based on studies that indicated that chromium compounds bound to insulin at non-physiologically relevant concentrations of chromium (although the authors themselves indicated the concentration discrepancy).[16] Most recently workers in this laboratory have isolated LMWCr from porcine kidney and porcine kidney powder; acid hydrolysis of these materials yields products quite similar to "GTF".[13] Consequently, "GTF" from mammalian sources is probably an artifact of acid hydrolysis. Why Brewer's yeast accumulates chromium and in what form it is accumulated are still unanswered questions.

Chromium Picolinate, $Cr(pic)_3$

The proposed identification of nicotinate (3-carboxypyridine) in "GTF" stimulated an interest in the synthesis of chromic nicotinate complexes.[17−20] The products of reaction of chromium(III) sources and the related pyridinecarboxylic acids picolinic acid (2-carboxypyridine) and isonicotinic acid (4-carboxypyridine) have also been studied in some detail.[21−23] Chromium(III) picolinate, $Cr(pic)_3$, has been the most thoroughly studied of these synthetic products and has become a very popular nutritional supplement; products containing $Cr(pic)_3$ generate over $100 million in sales annually as the supplement is available over-the-counter in numerous forms, including pills, chewing gums, sports drinks, and nutrition bars. $Cr(pic)_3$ is a relatively well absorbed form of chromium (2−5% efficiency compared to dietary chromium, which is only absorbed with approximately 0.5% efficiency) and has been proposed to be the biologically active form of chromium.[24] Yet, $Cr(pic)_3$ has not been shown to possess any intrinsic activity nor to occur naturally in mammals or other organisms, and there is no reason to expect it to exist naturally in vivo, especially as chromium and picolinic acid levels in tissues make its generation unlikely at best.

In 1995 questions arose about the use of $Cr(pic)_3$ as a dietary supplement as Wetterhahn and co-workers showed that the compound caused clasotogenic damage in Chinese hamster ovary cells.[25] Unfortunately, these studies used non-physiologically significant levels of chromium which cast doubt on their significance. Work done in this laboratory recently has shown that at physiologically relevant concentrations of chromium (120 nM) and of biological reductants such as ascorbic acid and thiols (5 mM), $Cr(pic)_3$ catalytically produces hydroxyl radicals, which can cleave DNA.[26] This ability stems from the combination of chromium and picolinate; the picolinate ligands shift the redox potential of the chromic center such that it is susceptible to reduction. The reduced chromous species interacts with dioxygen to produce reduced oxygen species including hydroxyl radical. These studies are in accord with earlier studies by Sugden and co-workers that showed that mutagenic forms of chromium(III) possessed chelating ligands containing pyridine-type nitrogens coordinated to the metal.[27] Recent studies have also shown that $Cr(pic)_3$ is remarkably stable in buffered aqueous solution[28] and in synthetic gastric fluid[29] and passes unhindered through the jejunum.[29] It is consequently probable that $Cr(pic)_3$ enters cells intact, i.e., in the potentially harmful form. The supplement has also been shown to not release its chromium efficiently to biological chromium-binding species such as apotransferrin[28] or apoLMWCr unless the metal is reduced to the chromous level;[30] thus, for the supplement to serve as a source of chromium, it must enter into the type of chemistry demonstrated to lead to the catalytic generation of hydroxyl radical.

In the past 10 years, a number of investigators have examined the effects of administering $Cr(pic)_3$ to rats on regular diets.[31−33] Detailed examinations of the effect of $Cr(pic)_3$ supplementation of the diet in amounts up to 1500 mg/kg diet for up to 24 weeks have observed no effects on body mass, percentage lean or fat content, tissue size (heart, testes, liver, kidney, muscle, epididymal fat, spleen, and kidney), or blood variables (fasting serum glucose, cholesterol, insulin, etc.), while no acute toxic effects were identified.[33] The lack of any beneficial effect on growth, fat content, or glucose, insulin, or cholesterol levels raises questions about the use of the compound as a therapeutic in non-chromium-deficient subjects. Studies are currently in progress in this laboratory to determine the stability, subcellular distribution, and concentration of $Cr(pic)_3$ in animals supplemented with the compound.

One problem in studies examining the stability and distribution of $Cr(pic)_3$ or other chromium pyridinecarboxylate compounds has been the lack of spectroscopic techniques to identify the materials. Researchers in this laboratory have been studying the use of paramagnetic NMR to examine potentially biologically relevant chromium(III) complexes.[34−36] This has led to the demonstration that through a combination of ^1H and ^2H NMR, all the nicotinate proton (deuteron) resonances of mononuclear and multinuclear chromium nicotinate complexes can be assigned.[37] Similarly, the ^1H and ^2H NMR spectra of this compound[37] and of the byproduct of its synthesis, $Cr_2(\mu\text{-}OH)_2(pic)_4$, have also been reported and assigned.[28] The electronic spectrum of $Cr(pic)_3$ has been a matter of debate for several decades; however, starting from the recent insights of Stearns and Armstrong,[22] workers in this laboratory have recently resolved these discrepancies.[28] Currently, an examination of the distribution and concentration of chromium picolinate in tissue and in cells

is underway to determine whether it remains intact and the potential significance of its DNA-cleaving ability.

Low-Molecular-Weight Chromium-Binding Substance

Insulin dose−response studies using rat adipocytes have indicated an intrinsic biological function for only one Cr-containing biomolecule, low-molecular-weight chromium-binding substance (LMWCr). Isolated rat adipocytes in the presence of LMWCr and insulin display an increased ability to metabolize glucose to produce carbon dioxide or total lipids; this increase occurs without a change in the insulin concentration required for half-maximal stimulation.[15,38] This lack of change in half-maximal insulin concentration suggests a role for LMWCr inside the insulin-sensitive cells after insulin binds externally to the insulin receptor.[15] The stimulation of glucose metabolism by LMWCr is proportional to its chromium content.[39] LMWCr is a naturally occurring oligopeptide composed of glycine, cysteine, aspartate, and glutamate with the carboxylates comprising more than half of the total amino acid residues.[40,41] Despite its small size (approximately 1500 molecular weight), the molecule tightly binds 4 equiv of chromic ions. The binding is quite tight ($K \approx 10^{21}$ M^{-4}) and highly cooperative (Hill coefficient, $n = 3.47$);[30] this indicates that essentially only apoLMWCr and holoLMWCr (Cr_4-LMWCr) coexist in solution. ApoLMWCr can accept chromic ions from biological molecules including Cr_2-transferrin.[30,42] Spectroscopic studies suggest that the chromic ions comprise an anion-bridged multinuclear assembly supported by carboxylates from the oligopeptide.[41] As holoLMWCr can be prepared simply by addition of chromic ions to solutions of apoLMWCr, these anionic bridges are probably oxide and/or hydroxide ions.[39,43,44] To date the oligopeptide has been isolated and purified from rabbit liver,[40] bovine liver,[41] porcine kidney,[13] and porcine kidney powder[13] and partially purified from dog[45] and mouse liver.[42] A related chromium-containing oligopeptide from bovine colostrum (M-LMWCr) is comprised of the same amino acids but in distinctly different ratios and also stimulates insulin-dependent glucose metabolism in rat adipocytes.[38] The significance of these differences between the liver and colostrum oligopeptides is essentially unexplored. Curiously, the oligopeptide is maintained in the soluble portion[42] of insulin-sensitive cells and the nucleus (J. Vincent and J. Ramirez, unpublished results) in the apo form. The oligopeptide is isolated as the holo-oligopeptide (so that it may be followed in the purification schemes by its chromium content), which means that an in vivo or in vitro chromium-loading step is required.[40,41] This observation has resulted in the suggestion that LMWCr may play a role in chromium detoxification; however, injection of chromic ions or chromate into mice does not stimulate the production of LMWCr.[42] Thus, while LMWCr does carry chromium into the urine after intake of large dosages of Cr(III) or Cr(VI),[45] the suggested detoxification role of LMWCr is unlikely to be its primary function.

LMWCr has been proposed to function as part of a unique autoamplification system for insulin signaling (Figure 1).[7,8] In this mechanism, apoLMWCr is stored in insulin-sensitive cells. In response to increases in blood insulin concentrations (as would result from increasing blood sugar concentrations after a meal), insulin binds to its receptor, bringing about a conformation change which results in the autophosphorylation of tyrosine residues on the internal side of the receptor. This transforms the receptor into an active tyrosine kinase and transmits the signal from insulin into the cell. In response to insulin, chromium is moved from the blood to insulin-sensitive cells. Here, the chromium flux results in the loading of apoLMWCr with chromium. The holoLMWCr then binds to the receptor, presumably assisting in maintaining the receptor in its active conformation, amplifying its kinase activity. When the signaling is to be turned off, a drop in blood insulin levels facilitates relaxation of the conformation of the receptor, and the holoLMWCr is excreted from the cell. Ultimately, LMWCr is efficiently excreted in the urine.

While insulin dose−response studies with LMWCr suggested a role inside the cells, studies by Yoshimoto and co-workers suggested that chromium functioned inside the cell during or before glucose transport.[46] Because the primary events between insulin binding to its receptor and glucose transport are signal transduction events, i.e., phosphorylation/dephosphorylation of protein residues, a role for LMWCr in these events has been probed in this laboratory. LMWCr has been shown to activate the tyrosine kinase activity of insulin-activated insulin receptor[43] and to activate a membrane phosphotyrosine phosphatase in adipocyte membranes.[44] For example, the addition of bovine liver LMWCr to rat adipocytic membranes in the presence of 100 nM insulin results in a concentration-dependent, up to 8-fold stimulation of insulin-dependent protein tyrosine kinase activity, while no activation of kinase activity is observed in the absence of insulin (Figure 2).[43] The dependence of the kinase activation on the concentration of LMWCr can be fit to a hyperbolic curve to give dissociation constants (K_m's) of approximately 875 pM, indicating extremely tight binding. Blocking the insulin-binding site on the external α subunit with antibodies whose epitope lies in this region results in the loss of the ability to activate insulin receptor kinase activity (Figure 3).[43] Examining the potential activation of isolated rat insulin receptor by bovine liver LMWCr in the presence of insulin indicates that LMWCr can amplify the isolated receptor protein tyrosine kinase activity by approximately 7-fold, conclusively demonstrating that the receptor is the site of interaction with LMWCr. Fitting the activation curve to a hyperbolic function gives a dissociation constant of approximately 250 pM.[43] The site of LMWCr binding on insulin receptor can be further refined. Studies with a catalytically active fragment (residues 941−1343) of the β subunit of human insulin receptor (which does not require insulin for kinase activity) reveal that LMWCr can stimulate kinase activity 3-fold with a dissociation constant of 133 pM (Figure 4). Thus, LMWCr binds at or near the

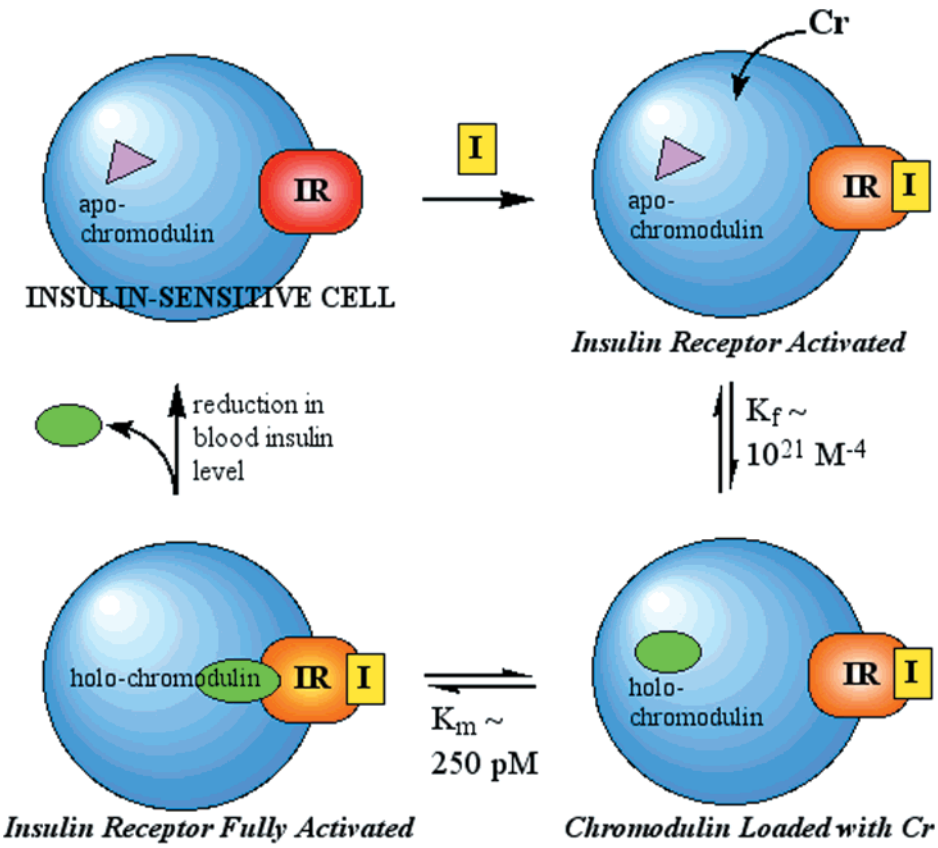

FIGURE 1. Proposed mechanism for the activation of insulin receptor activity by chromodulin (LMWCr) in response to insulin. The inactive form of insulin receptor (IR) is converted to the active form by binding insulin (I). This triggers a movement of chromium from the blood into insulin-dependent cells, which in turn results in apochromodulin (pink triangle) binding chromium. Finally, the holochromodulin (green oval) binds to insulin receptor, further activating the receptor kinase activity. Apochromodulin is unable to bind to receptor and activate kinase activity. When the insulin concentrations drops, holochromodulin is released from the cell to relieve its effects.

kinase active site.[47] Chromium plays a crucial role in the activation of insulin receptor kinase activity by LMWCr.[43] ApoLMWCr displays little ability to activate insulin-dependent tyrosine kinase activity in the rat adipocyte membranes, with the small amount of activity readily attributable to residual chromium. However, titration of apoLMWCr with chromic ions results in the total restoration of the ability to activate kinase activity; approximately four chromic ions per oligopeptide are required for maximal activity. This is consistent with the number of chromiums (four per oligopeptide) reported to be bound to holoLMWCr from liver sources. The activity of LMWCr is rapidly restored upon the addition of chromium, consistent with binding studies which indicate that Cr binding is highly cooperative, such that holoLMWCr forms rapidly to the exclusion of complexes of LMWCr without its full complement of chromium. This reconstitution of LMWCr's activation potential is specific to chromium. Transition metal ions other than chromium which are commonly associated with biological systems are ineffective in potentiating the ability of apoLMWCr to activate kinase activity (Figure 5). In fact, all the ions except Cr^{3+} resulted in loss of activation potential relative to apoLMWCr. Similarly, the metal ions themselves (in the absence of apoLMWCr) are ineffective in activating the insulin-dependent kinase activity. Thus, the ability of LMWCr to potentiate the effects of insulin in stimulating the insulin-dependent protein tyrosine kinase activity of insulin receptor is specific to chromium and is directly dependent on the chromium content of LMWCr.

In human euglycemic hyperinsulinemic clamp studies, Brian Morris and co-workers have shown that increases in blood insulin concentrations following an oral glucose load result in decreases in plasma chromium levels; a subsequent infusion of insulin led to further chromium losses.[48] Within 1.5 h after the increases of blood insulin concentrations, blood chromium levels started to recover. Patients also showed increased urinary chromium losses during the course of the experiments, with the amount of chromium lost roughly corresponding to the amount of chromium estimated to be lost from the intravascular space.[48]

Studies with human and rat tissue have demonstrated that chromium binding by insulin-dependent tissues is significantly enhanced by glucose, suggesting that chromium may translocate from the blood compartment to insulin-sensitive tissues.[49] Researchers in this laboratory have recently demonstrated in vivo that chromium is bound by LMWCr in the nucleus and cytosol of rat hepatocytes (which are insulin-sensitive) in response to insulin (J. Ramirez and J. B. Vincent, unpublished results).

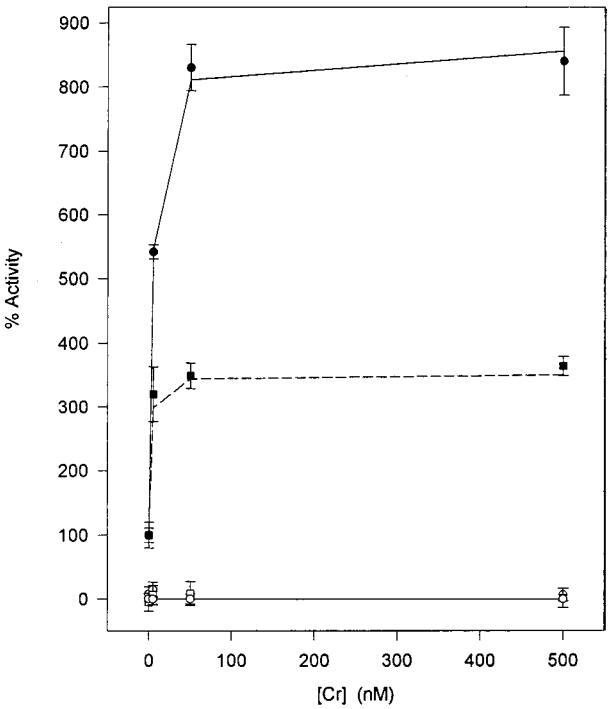

FIGURE 2. Activation of rat adipocytic membrane protein tyrosine kinase activity using 0.75 μM fragments of cell division kinase p34^{cdc2} (circles) and gastrin (squares) as substrates by bovine liver LMWCr in the presence (solid symbols) and absence (open symbols) of 100 nM insulin. 100% activity corresponds to insulin-stimulated kinase activity in the absence of LMWCr. Adapted from ref 43.

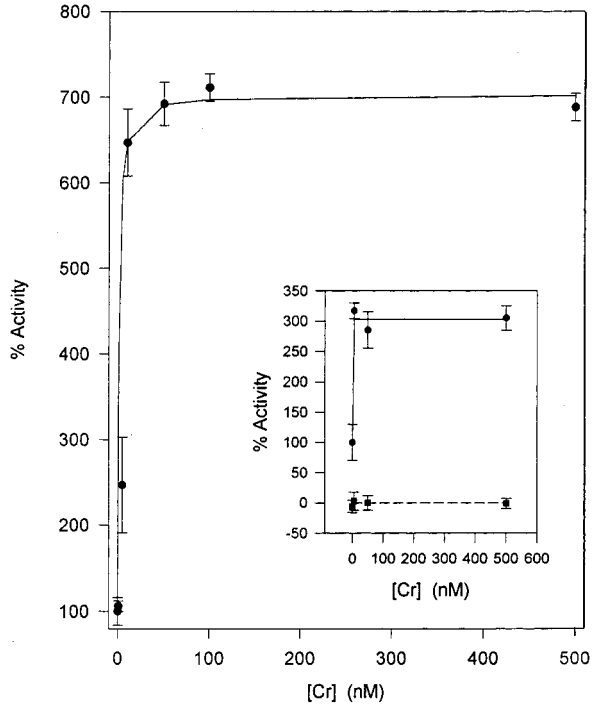

FIGURE 3. Activation of protein tyrosine kinase activity of isolated rat insulin receptor by bovine liver LMWCr using a fragment of gastrin as substrate (0.75 mM) in the presence of 100 nM insulin. The inset shows the protein tyrosine kinase activity of rat adipocytic membranes in the presence (squares) or absence (circles) of polyclonal antibodies whose epitope corresponds to amino acids 29−48 mapping at the amino terminus of the precursor form of human insulin receptor α chain using a fragment of gastrin (0.75 μM) as substrate in the presence of 100 nM insulin by LMWCr. Adapted from ref 43.

Numerous other studies have demonstrated that chromium is released in urine within 90 min of a stress such as sugar intake.[50−53] As glucose tolerance as a result of repeated carbohydrate stress decreases, the mobilization of chromium and resulting chromium loss have been shown to decrease.[53] Given that LMWCr appears to represent the major form of chromium in urine under certain conditions,[54] these homeostasis and urinary output studies indicate that chromium stored in the blood is mobilized in response to increases in blood insulin concentrations where ultimately it appears in the urine in the form of LMWCr.

The model for chromium function in carbohydrate metabolism also can explain some problematic aspects of chromium nutritional studies. Chromium appears to serve only as a nutritional supplement, not as a therapeutic;[55] only patients suffering from chromium deficiency are expected to have beneficial effects from dietary chromium supplementation. Thus, studies with $Cr(pic)_3$ (vide supra) and other forms of chromium nutritional supplements have no effect on the body mass or blood variables of healthy subjects. Treatment of rats with 20 mg of chromium as LMWCr per day for 12 weeks has recently been shown to have little if any effect on rats;[56] unfortunately (in terms of its use as a dietary supplement or therapeutic), LMWCr appears to be recognized and readily excreted. Injection of LMWCr into rabbits has been shown to lead to rapid excretion of chromium, especially compared to use of other forms of chromium; this is reflected in the mean tubular reabsorption rate for LMWCr of 23.5% in contrast to rates of 85.7 and 92.5% for chromate and chromium chloride, respectively.[45] This is probably also responsible for the extremely high LD_{50} for LMWCr injected into mice of 135 mg/kg of body mass.[42]

Given the unwieldy name of LMWCr and its proposed mechanism of action, workers in this laboratory would like to propose the name *chromodulin* for the oligopeptide. The basis of the name is the similarity of the mechanism of action to that of the calcium-binding protein calmodulin.[57] Both bind 4 equiv of metal ions in response to a metal ion flux; however, the four calcium ions which bind to the larger protein calmodulin rest in mononuclear sites. Both holoproteins selectively bind to kinases and phosphatases, stimulating their activity.

Synthetic Models

The recent research on chromodulin has inspired synthetic efforts to prepare new chromium carboxylate assemblies. Known assemblies with nuclearity greater than 2 (but less than 8) possess four types of cores: symmetric[58] and unsymmetric[59] Cr_3O, $Cr_3(OH)_2$,[60] and Cr_4O_2[61−63] (Figure 6). Numerous examples of the type containing the symmetric Cr_3O core have been well-characterized, and interest in these complexes dates back to the late 19th

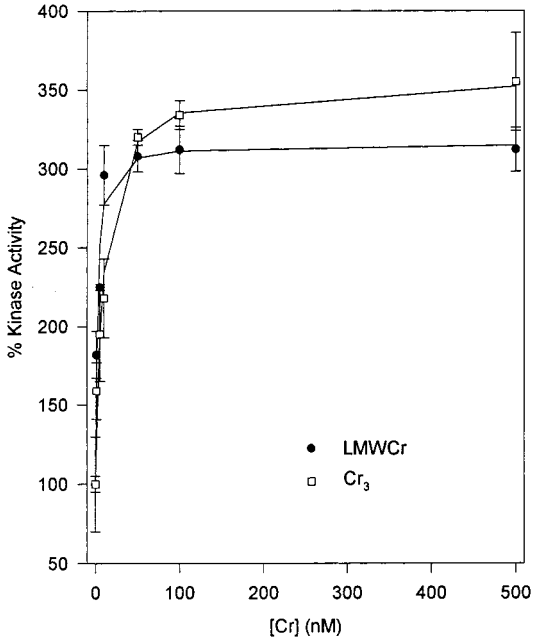

FIGURE 4. Activation of protein tyrosine kinase activity of the isolated active site fragment of the β subunit of human insulin receptor by bovine liver LMWCr (open squares) and $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$ (solid circles) using a fragment of gastrin (0.75 μM) as substrate. Adapted from ref 47.

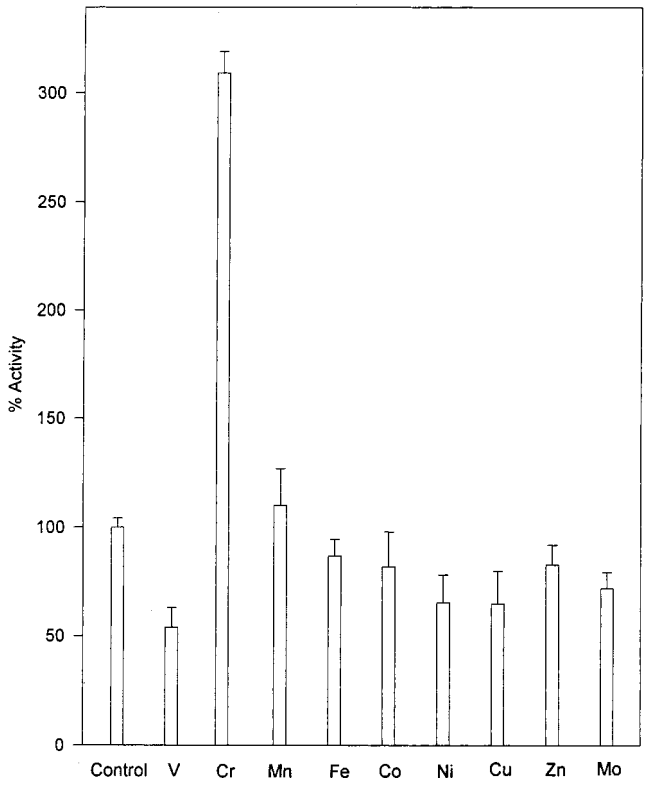

FIGURE 5. Activation of rat adipocytic membrane protein tyrosine kinase activity using 0.75 μM gastrin fragment as substrate by metal ions (500 nM) and apoLMWCr (125 nM) in the presence of 100 nM insulin. The control contains no added metal ion or apoLMWCr. Adapted from ref 43.

century.[58] The other cores have only been prepared during the past decade using the symmetric trinuclear complexes as starting materials.

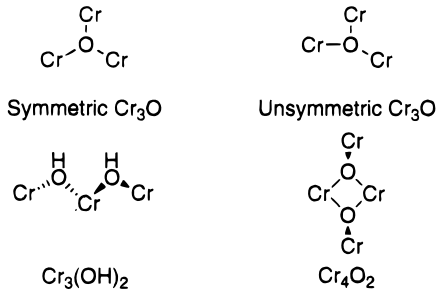

FIGURE 6. Cores of known tri- and tetranuclear anion-bridged chromic carboxylate assemblies.

Given the novel role in the autoamplification of insulin signal transduction for chromodulin and its rather simple composition (carboxylate-rich oligopeptide binding four chromic ions), attempts have been made to identify a functional model for chromodulin. Such a biomimetic would be required to be soluble and stable in aqueous solution. Few of the known trinuclear and tetranuclear Cr(III) oxo(hydroxo)-bridged carboxylate assemblies are soluble in water. On the basis of these requirements, two assemblies have been examined: $[Cr_3O(O_2CCH_3)_6(H_2O)_3]^+$ and $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$.[47] Both possess the symmetric basic carboxylate-type structure comprised of a planar triangle of chromic ions with a central μ_3-oxide. The acetate complex does not activate the tyrosine protein kinase activity of the active site fragment of insulin receptor or of adipocytic membrane fragments in the presence of insulin and actually inhibits the activity. In stark contrast, the propionate analogue activates the kinase activities in a fashion very similar to that of chromodulin. The kinase activity of the isolated receptor fragment, for example, is stimulated approximately 3-fold with a dissociation constant of 1.00 nM (Figure 4).[47] The complex appears to be a functional biomimetic for chromodulin and supports the existence of a multinuclear chromic assembly in chromodulin.

The propionate biomimetic has been found to have striking in vivo effects, lowering plasma triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol levels after 12 weeks of supplementation in rats at a level of 20 mg/kg of body mass daily and potentially lowering body mass and fat content.[56] No acute toxic effects were observed for supplementation with the compound, and it does not give rise to DNA damage as observed with $Cr(pic)_3$.[64] Thus, the propionate complex could have potential as a therapeutic. These results are also consistent with the proposed mechanism. In contrast to chromium supplements that only provide a source of chromium and have no intrinsic activity, a functional biomimetic (if it entered insulin-sensitive cells intact) could trap insulin receptor in its active conformation, amplifying insulin signaling and subsequent cellular activity. Efforts to determine the distribution and concentration of the biomimetic in rats and to develop a second generation of biomimetics and potential therapeutics are underway.

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